Binding Characteristics of Tumor Necrosis Factor Receptor-Fc Fusion Proteins vs Anti-Tumor Necrosis Factor mAbs

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Tumor necrosis factor (TNF) antagonists are efficacious in the treatment of various autoimmune diseases. Two classes of TNF antagonists are currently commercially available: soluble TNF receptor-Fc fusion proteins (etanercept) and anti-TNF mAbs (adalimumab and infliximab). The classes differ in molecular structures and mechanisms of action. The interactions between TNF antagonists with TNF molecules were characterized. The anti-TNF mAbs, but not the soluble TNF receptor, formed visible lines of precipitation in Ouchterlony assays. The molecular weights of complexes formed by TNF (52 kDa) with either etanercept (130 kDa), adalimumab (150 kDa), or infliximab (average 165 kDa) were determined by size exclusion chromatography-light-scattering assays. Etanercept and TNF formed complexes of 180 and 300 kDa, representing one and two etanercept monomers bound to a TNF trimer, respectively. Adalimumab and infliximab formed a variety of complexes with TNF with molecular weights as high as 4,000 and 14,000 kDa, respectively, suggesting the presence of complexes with a wide range of sizes and stoichiometries. The absence of large complex formation with the binding of soluble receptor-fusion proteins to TNF may account for the different clinical efficacy and safety profiles of the two classes of TNF antagonists.

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INTRODUCTION

Biologic therapies that target the proinflammatory cytokine tumor necrosis factor (TNF) have been shown to be efficacious in the treatment of several autoimmune diseases, including moderate to severe plaque psoriasis, moderate to severe rheumatoid arthritis, and psoriatic arthritis (Nash and Florin, 2005; Trent and Kerdel, 2005) (Enbrel® (etanercept) prescribing information; (2006). Immunex Corporation; Humira® (adalimumab) prescribing information (2005) Abbott Laboratories; Remicade® (infliximab) prescribing information (2006) Centocor Inc.). Some, but not all of the TNF antagonists are also effective in the treatment of granulomatous diseases, such as Crohn's disease and Wegener's granulomatosis (Targan *et al.*, 1997; Mukhtyar and Luqmani, 2005; Nash and Florin, 2005; Hanauer *et al.*, 2006). There are currently two classes of marketed biologic drugs that reduce TNF bioavailability: soluble TNF receptor-Fc fusion proteins (etanercept) and anti-TNF mAbs (adalimumab and infliximab).

Despite their common target, the two classes of agents differ markedly in molecular structure and mechanism of action (Calabrese, 2003). The anti-TNF mAbs bind TNF with high avidity, dissociate slowly, and have a long serum half-life (Scallon *et al.*, 2002; Nestorov, 2005). The soluble TNF receptor also binds TNF with high avidity, but reversibly and rapidly dissociates from TNF and has a shorter serum half-life (Scallon *et al.*, 2002; Nestorov, 2005). All three of the currently available TNF antagonists bear the Fc portion of human IgG1. Fc is a structural component of the mAbs, whereas it is genetically fused to the soluble receptor.

The biochemical nature and ability to form large protein complexes by a biologic agent that contains an Fc sequence may have profound impact on Fc-mediated functions. We investigated the interactions between the currently available TNF antagonists and their target, TNF.

RESULTS

Differences in diffusion characteristics between soluble receptors and mAbs

In Ouchterlony double-diffusion assays, all of the agents formed detectable immunoprecipitates with antibodies directed against the Fc components (Figure 1). Similarly, interactions between the anti-TNF mAb and TNF resulted in the formation of large, precipitable complexes. In contrast,

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Abbreviations: SEC-LS, size exclusion chromatography-light scattering; TNF, tumor necrosis factor; TNF_t, tumor necrosis factor trimer

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etanercept-TNF complexes did not form precipitation lines in agarose gels.

Physical properties and stoichiometry of TNF-etanercept complexes

The molecular weights of etanercept, TNF, and TNF-etanercept complexes were determined by size exclusion chromatography-light scattering (SEC-LS). Etanercept and TNF trimers (TNF_t) were calculated to be 130 and 52 kDa, respectively (Table 1).

Etanercept was found to exist in two distinct stable complexes with TNF (Table 1 and Figure 2). Complex I consisted of one etanercept molecule bound to a single TNF_t, with an apparent molecular weight of 180 kDa, which formed when TNF was in excess. Two etanercept molecules bound to a single TNF_t comprised Complex II, which had a molecular weight of 300 kDa and formed when etanercept was in excess.

Differences in complex formation with TNF between soluble receptors and mAbs

Both adalimumab (Figure 3) and infliximab (Figure 4) formed large protein complexes with TNF, as determined by SEC-LS analysis. The molecular weight of adalimumab- and infliximab-TNF complexes ranged up to 4,000 and 14,000 kDa, respectively. In contrast, etanercept did not form large complexes with TNF (Figure 5).

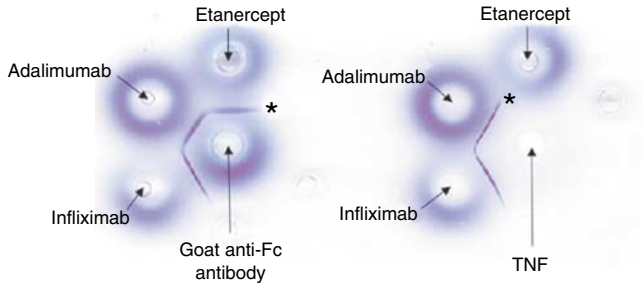

Figure 1. **Precipitation of protein complexes formed by TNF antagonists and TNF.** Samples were applied to rosette wells of Ouchterlony agarose gels, with control or test samples in the center wells and TNF antagonists in the outside wells. The left panel represents control reactions between the TNF antagonists and anti-Fc antibodies, and the right panel shows the interactions between the TNF antagonists and TNF. *Precipitation lines.

Table 1. Physical properties of etanercept, TNF and complexes

	MW (kDa)	R_h (nm)
Etanercept	130	7.1
TNF_t	52	3.1
Etanercept-TNF_t complex I	180	6.8
$Etanercept_2$-TNF_t complex II	300	9.1

$Etanercept_2$, two etanercept molecules; MW, molecular weight; R_h, hydrodynamic radius; TNF_t, tumor necrosis factor trimer.

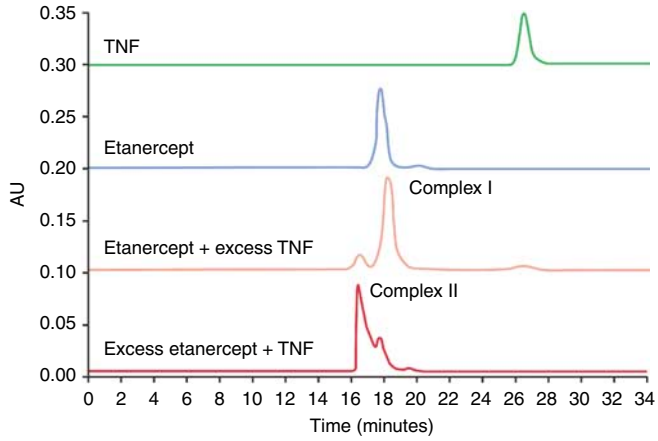

Figure 2. **Stoichiometry of etanercept-TNF complexes.** Etanercept, TNF, and mixtures of preincubated etanercept and TNF at ratios of 1:3 and 3:1 were separated by size exclusion chromatography and the retention time of each sample was examined. AU = absorbance units.

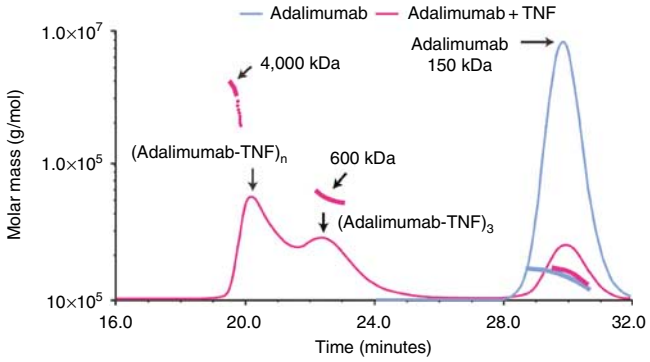

Figure 3. **Analysis of adalimumab-TNF complexes.** Adalimumab and a mixture of adalimumab and TNF (3:1 ratio) were analyzed by SEC-LS. The lighter lines represent the trace of proteins and complexes absorbing at 280 λ. The heavier lines represent molecular masses of complexes detected by light scattering.

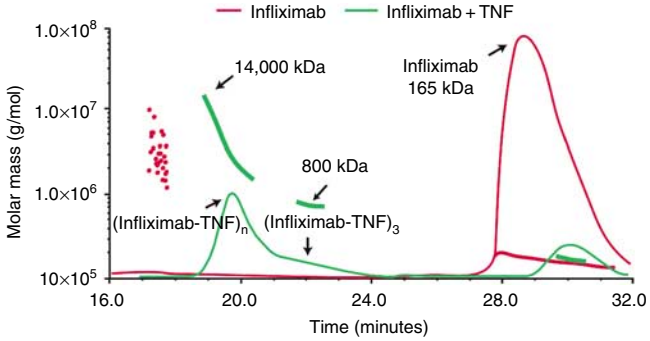

Figure 4. **Analysis of infliximab-TNF complexes.** Infliximab and a mixture of infliximab and TNF (3:1 ratio) were analyzed by SEC-LS. The lighter lines represent the trace of proteins and complexes absorbing at 280 λ. The heavier lines represent molecular masses of complexes detected by light scattering. The red dots represent trace amounts of high-molecular weight aggregates of infliximab.

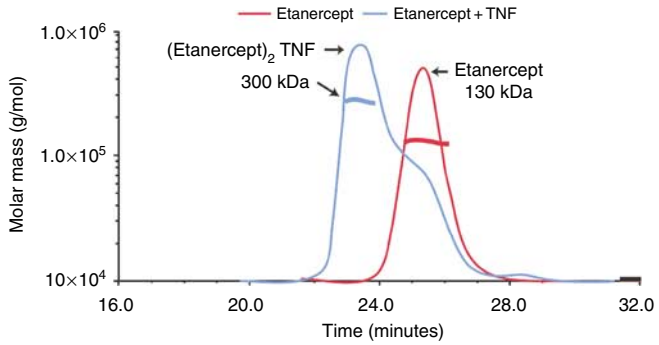

Figure 5. **Analysis of etanercept-TNF complexes.** Etanercept and a mixture of etanercept and TNF (3:1 ratio) were analyzed by SEC-LS. The lighter lines represent the trace of proteins and complexes absorbing at 280 λ. The heavier lines represent molecular masses of complexes detected by light scattering.

DISCUSSION

The soluble TNF receptor, etanercept, did not form large precipitable complexes with TNF in Ouchterlony double-diffusion assays; in contrast, interactions between the anti-TNF mAbs and TNF led to the formation of large complexes. The mAbs typically do not form precipitable complexes in these types of assays, as they all bind to the same portion of the target molecule. Our data therefore suggest that the anti-TNF mAbs crosslinked via TNF to form the precipitates seen in the double-diffusion assays. Experiments to further elucidate the binding characteristics of the TNF antagonists to TNF were subsequently performed.

Using SEC-LS methodology, the molecular weight of etanercept was found to be 130 kDa. In agreement with published studies, TNF was found to exist as a trimer (TNF_t) with a molecular weight of 52 kDa (Eck and Sprang, 1989). Etanercept was determined to form two types of complexes with TNF_t: as a single molecule bound to a single TNF_t (Complex I) or as two etanercept molecules bound to a single TNF_t (Complex II). Infliximab has been shown to bind to both monomeric TNF and TNF_t (Scallon *et al.*, 2002). Although adalimumab (150 kDa) is similar in size to etanercept (130 kDa), this anti-TNF mAbs formed various complexes up to a molecular weight of 4,000 kDa, representing numerous stoichiometries.

The SEC-LS analyses of the complexes formed between the TNF antagonists and TNF were consistent with the results of the immunodiffusion assays. Etanercept, which failed to immunoprecipitate TNF in double-diffusion assays, formed small complexes with TNF in SEC-LS analyses. In contrast, the anti-TNF mAbs formed large complexes in both analyses. Based on the size of the etanercept-TNF complexes and the lack of immunoprecipitation, we hypothesize that the flexibility of etanercept is more restricted, possibly resulting in some steric hindrance that prevents crosslinking of multiple TNF_t. The observation that the hydrodynamic radius of etanercept-TNF Complex I was smaller than the hydrodynamic radius of etanercept alone suggests that this complex is particularly compact in nature. The flexibility of the mAbs apparently allowed crosslinking of several antibodies to a soluble TNF_t.

Activation of complement-dependent cytotoxicity (via the classical complement pathway) or antibody-dependent cellular cytotoxicity (via Fcγ receptors) requires multiple Fc regions in close physical proximity to each other (Segal *et al.*, 1983). Thus, large complexes may allow crosslinking of the Fcγ receptors and increased binding to C1q, which may lead to antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, respectively, *in vivo*. Adalimumab (Humira® (adalimumab) prescribing information (2005) Abbott Laboratories) and infliximab (Remicade® (infliximab) prescribing information (2006) Centocor Inc.) have been shown to lyse TNF-expressing cells *in vitro*.

The major differences between the actions of the two classes of drugs *in vivo* appear to be related to effects on granulomatous diseases and infections. Both adalimumab and infliximab are efficacious in the treatment of Crohn's disease (Targan *et al.*, 1997; Hanauer *et al.*, 2006), and infliximab has been used in the treatment of Wegener's granulomatosis, (Mukhtyar and Luqmani, 2005) whereas etanercept has not demonstrated clinical benefits in these diseases. Another example of evidence for differences in mechanisms of action is the observation that patients with rheumatoid arthritis can be effectively switched from etanercept therapy to treatment with mAbs, and vice versa, with clinical benefit (Gomez-Reino and Carmona, 2006). Although all of the drugs target TNF, the interactions with TNF and subsequent effector mechanisms may differ sufficiently to allow a patient who is non-responsive to one drug to respond to the other class of TNF antagonists.

TNF is an important mediator in the immune response to granulomatous infections, and total blockade of this inflammatory cytokine has been postulated to be detrimental (Nestorov, 2005). It has been reported that the anti-TNF mAbs may be cytotoxic to TNF-producing cells) (Humira® (adalimumab) prescribing information (2005) Abbott Laboratories and Remicade® (infliximab) prescribing information (2006) Centocor Inc.) by inducing antibody-dependent cellular cytotoxicity (Scallon *et al.*, 1995), complement-dependent cytotoxicity (Scallon *et al.*, 1995), or apoptosis (Scallon *et al.*, 2002; Shen *et al.*, 2005a, b, 2006), which may explain clinical differences between soluble receptors and mAbs (Haraoui, 2005). Further studies are required to determine the ramifications of these functional differences between the TNF antagonists observed in this study. The observed differences may help to explain differences in clinical efficacy and safety (Wallis *et al.*, 2004a, b, 2005).

MATERIALS AND METHODS

Reagents

Etanercept (Enbrel®; Amgen Inc., Thousand Oaks, CA) (Enbrel® (etanercept) prescribing information. (2006). Immunex Corporation) was supplied in a buffer containing 25 mM sodium phosphate, 25 mM L-arginine, 98 mM sodium chloride, and 1% sucrose. Adalimumab (Humira®; Abbott Laboratories, North Chicago, IL) (Humira® (adalimumab) prescribing information (2005) Abbott Laboratories) was supplied in 105 mM sodium chloride, 5.5 mM monobasic sodium phosphate dihydrate, 8.6 mM dibasic sodium phosphate dihydrate, 1 mM sodium citrate, 6.2 mM citric acid monohydrate, 66 mM

mannitol, 0.1% polysorbate 80, pH 5.2. Infliximab (Remicade®; Centocor Inc., Malvern, PA) was supplied in 1.6 mM monobasic sodium phosphate monohydrate, 3.4 mM dibasic sodium phosphate dehydrate, 5% sucrose, 0.005% polysorbate 80, pH 7.2. Purified recombinant TNF was supplied by Amgen Inc.

Ouchterlony double-diffusion assays

The central well of a rosette pattern of wells in a flat-bed agarose-polyethylene glycol Micro-Ouchterlony plate (MP Biomedicals, LLC, Aurora, OH) was loaded with a control sample of 10 μl (2 mg/ml) goat anti-human Fc polyclonal antibodies (Sigma Chemical Co, St Louis, MO) or 10 μl (0.89 mg/ml) recombinant human TNF. Samples of TNF antagonists (10 μl at 1.00–1.68 mg/ml) were applied to wells surrounding the center wells, and allowed to diffuse at 37°C for 4 hours in humid conditions, then at room temperature for 6 hours. The Micro-Ouchterlony plate was removed from the case and incubated under water at room temperature for 3 hours with several changes of water. Protein complexes formed by interactions between drug and control or target was visualized by staining the gels with Coomassie R250 dye and destaining (Invitrogen Corporation, Carlsbad, CA).

SEC-LS

Samples of individual TNF antagonists (100 μg) or mixtures of individual TNF antagonists with different molar ratios of TNF (1:3 or 3:1) were preincubated at 4°C for 16 hours or 37°C for 30 minutes to 16 hours and then applied to HPLC columns for separation by size. Experiments were performed on an Agilent 1100 HPLC system with a Superdex-200 gel filtration column (Amersham Pharmacia, Waukesha, WI). The samples were then passed through a Wyatt miniDawn LS laser light-scattering detector and Wyatt Optilab DSP Refractometer (Wyatt Technology Co, Santa Barbara, CA) to determine the molecular mass and radius of the protein complexes. Phosphate-buffered saline (2.67 mM potassium chloride, 138 mM sodium chloride, 1.47 mM potassium phosphate monobasic, 8.1 mM sodium phosphate dibasic, pH 7.4) was used as the mobile phase at 0.4 ml/minute. To confirm the results and to determine the hydrodynamic radius, additional experiments were also performed on a GPS solvent/sample module (GPCmax) with a triple detector array (TDA302, Visotek Co, Houston, TX).

CONFLICT OF INTEREST

All authors are all employees of Amgen Inc. All authors have stock or stock options.

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